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Presence of Muscarinic Acetylcholine Receptors in the Cattle Tick *Boophilus microplus* and in Epithelial Tissue Culture Cells of *Chironomus tentans*†

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Abstract: A muscarinic acetylcholine receptor (mAChR) has been demonstrated and partially characterized in larvae of the cattle tick *Boophilus microplus*. Its properties are compared with mAChR from an epithelial cell line from the dipteran insect *Chironomus tentans*. Competition studies with cholinergic ligands of different specificity revealed the muscarinic nature of the cholinergic receptors investigated in both species. In homogenates from tick larvae, specific binding sites for [^{3}H]quinuclidinyl benzilate (QNB) with high affinity (1·2 ± (0·13) nM; B_{max} 22·5 pmol mg protein^{-1}) were detected that do not bind nicotinic compounds specifically. The estimated IC_{50} values for nicotine, imidacloprid and α-bungarotoxin were all in the mM range. Additionally, with tick larvae, high-affinity nicotinic binding sites were detected with [^{3}H]nicotine which could be displaced by high concentrations of imidacloprid or QNB. The estimated IC_{50} values for nicotine, α-bungarotoxin, imidacloprid and QNB were 43(±8) nM, 0·8(±0·2) μM, 2·8(±0·6) μM and 78(±1·9) μM, respectively.

With homogenates of the non-neuronal insect cell line from *C. tentans*, only high-affinity binding sites for [^{3}H]QNB were found. Muscarinic antagonists selectively displaced [^{3}H]quinuclidinyl benzilate (QNB) binding to tick larvae homogenates. The mAChR of *B. microplus* preferred pirenzepine (IC_{50} 2·13(±1·02) μM) among different subtype-specific mAChR antagonists (4-DAMP had IC_{50} 49·9(±9·13) μM and methoctramine had IC_{50} 121(±14·2) μM) indicating a type of binding site similar to the vertebrate M1 mAChR subtype. The tick muscarinic receptor seems to be a G-protein-coupled receptor, as concluded from the 4·8-fold reduction in receptor affinity for binding of the muscarinic agonist oxotremorine M upon treatment with the non-hydrolysable GTP-analogue γ-S-GTP. Binding data for the agonists oxotremorine M (IC_{50} 71·3(±19·6) μM) and carbachol (IC_{50} 253(±87·1) μM) parallel the biological efficacy of these compounds, in that, while oxotremorine M showed some activity against ticks, carbachol was ineffective.

Key words: muscarinic acetylcholine receptor, cattle tick, insect, non-neuronal cell line

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1 INTRODUCTION

Activation of acetylcholine receptors (AChR), widespread in the animal kingdom, is involved in the transmission of neuronal signals. Two different classes of acetylcholine receptors, nicotinic and muscarinic (mAChR), have been identified throughout animal phyla, including insects.[1,2] The vertebrate nicotinic acetylcholine receptor has been characterized as a pentameric ligand-triggered cationic ion channel with a central pore.[3] Nicotinic acetylcholine receptors have been exploited as an insecticidal target with the insecticides nicotine and cartap (a nereistoxin analogue) and recently, and more successfully, with the introduction of a chloronicotinyl compound, the nicotinic agonist imidacloprid.[4,5]

In contrast to nicotinic AChRs, muscarinic AChRs are members of a family of GTP-binding integral membrane receptors and show considerable homology with adrenergic receptors and rhodopsins.[6,7] Recently, the functions of neuronal mAChR in insect CNS were discussed with respect to the synaptic location of the mAChR.[8] According to this study, the presynaptic mAChR, which is related to the vertebrate M2 mAChR, inhibits the acetylcholine release in sensory neurons, possibly by inhibition of cAMP or by activation of hyperpolarizing K^+ conductance. By contrast, postsynaptic mAChR, which is similar to vertebrate M1 and M3 mAChR, causes a prolonged depolarization and enhanced tendency to generate spikes in interneurons and motoneurons by a variety of ionic mechanisms which, in some cases, involve K^+ or Na^+ depolarization, possibly under the control of Ca^{2+} entry.[8]

Biological efficacy of muscarinic agonists against the cattle tick *Boophilus microplus* Can. was described in 1976 and it has been concluded that such compounds should have potential as new acaricides.[9] Recently, the presence of muscarinic binding sites has been demonstrated in mites,[10] but, up to now, no mAChR has been described in the tick *Boophilus microplus* Can.

Recent evidence suggests a further function of a muscarinic cholinergic system in addition to its role in synaptic transmission, in that the muscarinic cholinergic system appears to be involved in the regulation of development. Acetylcholine esterase activity as part of the cholinergic system was demonstrated to be expressed transiently in vertebrate and crustacean tissues during cell division and differentiation.[11,12] In addition, there was evidence for the presence of a muscarinic acetylcholine receptor in undifferentiated cells of vertebrate embryos.[13–15] Incubations with muscarinic antagonists led to abnormal gastrulation, which is indicative of involvement of mAChR in differentiation processes.[15]

Acetylcholine esterase activity has also been demonstrated in a *Chironomus tentans* Walker cell line and shown to be stimulated upon incubation with insect moulting hormone.[16] This cell line is characterized by its susceptibility to incubation with physiological concentrations of 20-hydroxyecdysone. Hormone-dependent regulation of DOPA decarboxylase and chitin synthesis, as well as degradation, have been shown for these cells, which can therefore be designated as of epidermal or imaginal disc origin.[16] Muscarinic acetylcholine receptors have been characterized recently both pharmacologically and physicochemically.[17] Furthermore, *C. tentans* mAChR has been demonstrated by immuno staining with antibodies raised against vertebrate mAChR on *C. tentans* cells upon 20-hydroxyecdysone treatment.[18]

This paper reports on the physicochemical properties of an acetylcholine receptor of solely muscarinic pharmacology present in the cattle tick *B. microplus* and compares these data to those for mAChR in the epithelial *C. tentans* cell line. Comparison of acaricidal and insecticidal efficacy of cholinergic compounds is also presented to support conclusions on the different efficacies of nicotinic and muscarinic compounds on acari and insects. Both insect cells and tick larvae can be used as model systems for screening of muscarinic effectors with arthropodicidal efficacies.

2 EXPERIMENTAL METHODS

2.1 Cell culture

The epithelial cell line from *C. tentans* was cultured as described by Wyss.[19] Cells were kept at 25°C in Erlenmeyer flasks and subcultured after 12–14 days.

2.2 Tick larvae

Ticks (*B. microplus*) were bred on cattle and engorged adult female ticks were collected and incubated at 27°C and 85% RH until egg-laying and hatching of larvae was complete (four weeks). Larvae, 10–20 days old, were used for biological studies or frozen at −80°C for membrane preparations. Adult fed female ticks were used for injection bioassays after they had dropped off their host.

2.3 Chemicals

Muscarinic antagonists QNB ((±)-quinuclidinyl benzilate), 4-DAMP (4-diphenylacetoxy-*N*-(2-chloroethyl)piperidine hydrochloride), methoctramine (*N,N'-bis*[6-[[(2-methoxyphenyl)methyl]amino]hexyl]octane-1,8-diaminetetra-hydrochloride), pirenzepine (5,11-dihydro-11-[(4-methyl-1-piperazinyl)acetyl]-6*H*-pyrido-[2,3-*b*][1,4]benzodiazepin-6-one dihydrochloride) and the muscarinic agonists carbachol (carbamoylcholine

chloride), oxotremorine M (*N,N,N*,-trimethyl-4-(2-oxo-pyrrolidin-1-yl)but-2-ynammonium iodide) and bethanechol (carbamyl-β-methylcholine chloride) were purchased from RBI (Natick, MA, USA). Radiochemicals were obtained from DuPont NEN, Boston, MA, USA, ([^{3}H]QNB, 1·6 TBq mmol^{-1}; [^{3}H](−)-nicotine, 2·4 TBq mmol^{-1}) and from Amersham Buchler, Braunschweig, Germany ([^{3}H]α-bungarotoxin, 2·2 TBq mmol^{-1}). [^{3}H]Imidacloprid; 1·4 TBq mmol^{-1}) was custom synthesized by Amersham International plc (Buckinghamshire, UK). All other chemicals were of the purest grade available.

2.4 Protein determinations

Protein concentrations were determined according to the Bradford microassay using bovine serum albumin as a standard.[20]

2.5 Membrane preparations

2.5.1 C. tentans homogenates

These were prepared essentially according to Wegener *et al.*[17]

2.5.2 B. microplus homogenates

Frozen *B. microplus* larvae were homogenized prior to use in a phosphate buffer medium consisting of: potassium phosphate 100 mM; sucrose 250 mM; pH 7·0, freshly supplemented with the following protease inhibitors (μg ml^{-1}); leupeptin (10), aprotinin (10) and pepstatin (5). An Ultraturrax homogenizer (IKA, Germany) was used at 0°C with 10 ml buffer g^{-1} tick larvae. The homogenate was filtered through cheesecloth and the filtrate was pelleted by centrifugation (20000*g*; 4°C; 20 min). Pellets were re-suspended in phosphate buffer and homogenized in a glass Teflon potter. This homogenate was diluted to give 0·5 to 0·8 mg protein ml^{-1} and the suspension was stored on ice for further use.

2.6 Binding assays

Crude homogenates cooled in ice were incubated (30–60 min) in phosphate buffer containing the cholinergic ligands. The concentration of muscarinic ligand [^{3}H]QNB was adjusted to 2·5 nM in standard assays (concentrations and type of radioligand in other cases are given in the legends to tables and figures) and this 'total' binding was displaced by addition of unlabelled competitor in parallel incubation assays. Specific binding was calculated by subtracting non-specific from total binding values. Non-specific adsorption of ligands to filters did not exceed 260 dpm and was subtracted routinely. Three replicate determinations were performed in each case.

Incubations were terminated by rapid filtration through Whatman GF/C glassfibre filters followed by three washing steps with ice-cold buffer minus sucrose and protease inhibitors but containing ethanol (20 ml litre^{-1}). Filters were placed in vials, scintillation cocktail (Optifluor, Packard; 5 ml) was added and the radioactivity retained on the filters was determined with a Phillips PW 4700 liquid scintillation counter with a 50% counting efficiency for tritium.

Binding data were analyzed with the non-linear least squares parametric curve-fitting program Sigma Plot (Jandel Scientific). K_i values were calculated from IC_{50} values according to Cheng and Prusoff.[21]

2.7 Bioassays

2.7.1 Larvae packet test

B. microplus larvae (10–20 days old; 50–100) were transferred onto a 10 × 10 cm filter paper (No. 1573, Schleicher and Schuell) which had been treated 24 h previously with the desired concentration of the test compound diluted in a pure olive oil formulation. The filter papers were folded and closed with clamps to prevent larvae from escaping and these larvae 'packets' were incubated at 27°C and 85% RH for seven days. The number of dead tick larvae was determined after one and seven days and results were compared with those for larvae reared on filter papers treated with olive oil alone.

2.7.2 Injection assay

For each concentration tested the compound (1 μl in dimethylsulfoxide (DMSO) solution) was injected into the abdomen of each of five engorged female ticks. Egg laying was allowed to proceed for one week and eggs were then incubated (27°C; 85% RH) for three weeks, after which the number of larvae which hatched was recorded and compared with that for ticks injected with DMSO alone.

3 RESULTS

The results of analyses for binding of cholinergic ligands in homogenates of *B. microplus* larvae and *C. tentans* cell line, summarized in Table 1, indicate the specificity of the [^{3}H]QNB binding site to be exclusively muscarinic. Binding studies with the nicotinic agonist [^{3}H]nicotine revealed an additional nicotinic acetylcholine receptor in the tick (Fig. 1(a)), but not in the non-neuronal insect cell line (data not shown). [^{3}H]Nicotine binding was displaced by nicotine, α-bungarotoxin, imidacloprid and QNB with IC_{50} values of 43(±8) nM, 0·8(±0·2) μM, 2·8(±0·6) μM and

TABLE 1
Binding of Cholinergic Ligands to *Boophilus microplus* and *Chironomus tentans* Homogenates[a]

Species	*Labelled cholinergic ligand*	*Competitor (% of control) (±SEM)*		
		α-Bungarotoxin	*Imidacloprid*	*QNB*
B. microplus	[^{3}H]QNB[e]	113·7 (±18·8)[b]	104·2 (±13·4)[b]	14·2 (±2·1)[c]
	[^{3}H]imidacloprid[f]	93·4 (±21·9)[b]	97·8 (±9·4)[b]	80·9 (±4·2)[d]
C. tentans	[^{3}H]QNB[g]	106·1 (±6·5)[b]	112·9 (±1·1)[b]	18·6 (±1·6)[d]
	[^{3}H]imidacloprid[h]	85·9 (±2·8)[b]	96·3 (±4·0)[b]	89·0 (±8·1)[b]
	[^{3}H]α-bungarotoxin[i]	96·7 (±11·3)[b]	95·3 (±7·0)[b]	114·0 (±6·2)[b]

[a] Cell homogenates were prepared as described in the Experimental Methods section and incubated with the respective tritiated cholinergic ligands. This total binding was set to be 100% control binding. In parallel, unlabelled ligands (25 μM) were added as indicated above. Reduction of total binding compiled in the table is indicative of specific binding. $n = 4$, standard deviation given in the table.
[b] No specific binding of statistical significance.
[c] Specific binding ($P < 0.01$).
[d] Specific binding ($P < 0.1$); control: 100% [^{3}H]ligand binding.
[e] 2589 dpm mg^{-1} protein at 2·5 nM [^{3}H]QNB.
[f] 229 dpm mg^{-1} protein at 5 nM [^{3}H]imidacloprid.
[g] 5909 dpm mg^{-1} protein at 2·5 nM [^{3}H]QNB.
[h] 773 dpm mg^{-1} protein at 5 nM [^{3}H]imidacloprid.
[i] 5250 dpm mg^{-1} protein at 10 nM [^{3}H]α-bungarotoxin.

78(±2) μM, respectively. No specific nicotinic binding sites could be detected in *C. tentans* cell homogenates upon incubation with the radiolabelled nicotinic antagonist α-bungarotoxin or the nicotinic agonist imidacloprid (Table 1). Furthermore, the nicotinic antagonist α-bungarotoxin and the nicotinic agonists nicotine and imidacloprid were unable to displace [^{3}H]QNB binding effectively (Figs 1(b) and (c)), whereas typical muscarinic antagonists such as QNB (IC_{50} tick larvae 0·05(±0·02) μM; IC_{50} insect cells 0·3(±0·04) μM) and atropine (IC_{50} tick larvae 0·29(±0·09) μM; IC_{50} insect cells 7·59(±3·12) μM) did displace [^{3}H]QNB binding (Figs 1(a) and (b), Table 2). Treatment with antagonists discriminating between three vertebrate mAChR subtypes revealed the following sequence of affinity for mAChR from *B. microplus* larvae: pirenzepine (M1 subtype-specific) IC_{50} 2·13(±1·02) μM > 4-DAMP (M3 subtype-specific) IC_{50} 49·9(±9·13) μM > methoctramine (M2 subtype-specific) IC_{50} 121(±14·2) μM. Figure 1(d) shows [^{3}H]QNB displacement curves with tick homogenates and the muscarinic agonists oxotremorine M (IC_{50} 71·2(±19·6) μM) and carbachol (IC_{50} 253(±87·1) μM). Since mAChR are G-protein-coupled receptors, it should be possible to change the proportions of active and desensitized mAChR by the addition of non-hydrolysable GTP analogues.[22] Incubation of tick larvae homogenates with oxotremorine M in the presence of γ-*S*-GTP resulted in a displacement curve with an IC_{50} value of 340(±47·4) μM (Fig. 1(d)).

Specific binding of [^{3}H]QNB to crude homogenates of *B. microplus* larvae is saturable (Fig. 2(a)). As calculated from the saturation binding curve, maximum binding occurred above 25 nM. Scatchard analysis[23] of [^{3}H]QNB binding to *C. tentans* cell homogenates is presented in Fig. 2(b). Transformation of data according to the method of Chamness and McGuire[24] revealed a high-affinity binding component for QNB with an equilibrium dissociation constant K_D of 1·2 nM and a B_{max} of 22·5 fmoles receptor mg^{-1} protein. A Hill coefficient of 1·02 can be calculated from the slope of the logit-log transformation of the saturation curve (inset Fig. 2(b)).

An additional criterion for receptor characterization, besides ligand specificity and affinity, is the protein nature of the binding site. Treatment with 0·5 mg ml^{-1} activated proteinase K resulted in 55% and 68% loss in specific [^{3}H]QNB binding to *C. tentans* and *B. microplus* homogenates respectively (data not shown).

In order to further characterize [^{3}H]QNB binding in tick larvae homogenates, kinetic studies were carried out, the results of which are shown in Figs 3(a) and (b). Dissociation rate constants were determined as described in the legend (Fig. 3(a)) and linearization of the data is shown in the inset; the negative slope of the linearized plot defines K_{diss} as $2{\cdot}87 \times 10^{-2}$ min^{-1}. Determination of the association rate constant K_{ass} was carried out as described in the legend to Fig. 3(b) and the data were linearized as shown in the inset to this figure. K_{ass}, calculated from $[K_{obs}$ (0·1 min^{-1}) $- K_{diss}$ ($2{\cdot}87 \times 10^{-2}$ min^{-1})] [ligand concentration (2·475 nM)]$^{-1}$, was $2{\cdot}89 \times 10^7$ M^{-1} min^{-1}. Table 3 compares kinetic and equilibrium data from *B. micro-*

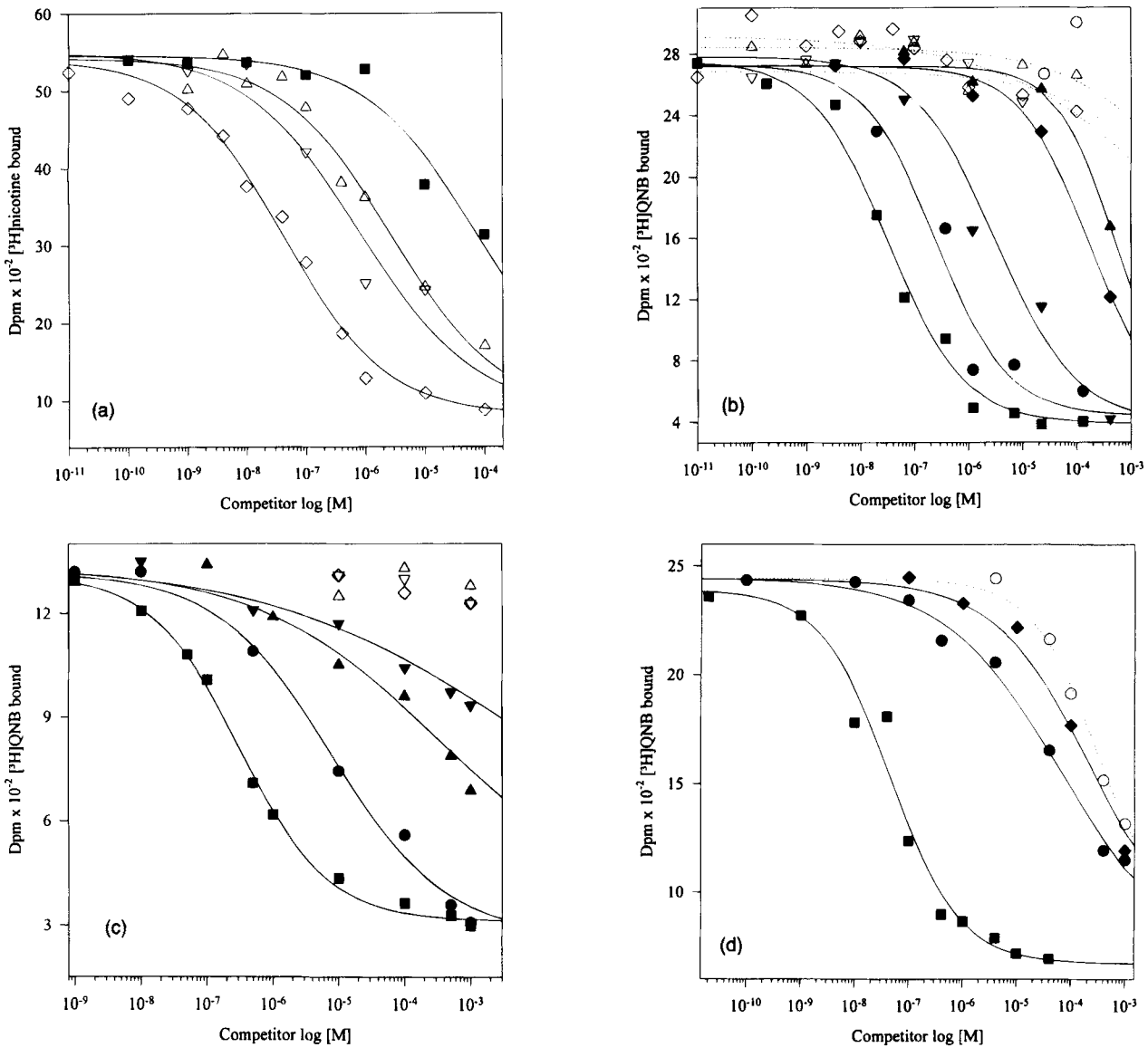

Fig. 1. Displacement of [^{3}H]cholinergic ligand binding to homogenates of *Boophilus microplus* larvae or *Chironomus tentans* cells by muscarinic and nicotinic antagonists. Homogenates were prepared and incubated as described in the text. Graphs show displacement of radioligand binding given as dpm with increasing concentrations of the respective muscarinic (closed symbols) and nicotinic (open symbols) antagonists. $n = 3$. Standard deviations did not exceed 20% for *d*-tubocurarine and α-bungarotoxin and 12% for all other ligands. Displacement curves were calculated from a four-parameter logistic curve fitting program (Sigma Plot, Jandel Scientific). (a) Displacement of [^{3}H]nicotine binding to homogenates of *B. microplus* larvae by nicotinic and muscarinic antagonists and nicotinic agonists. [^{3}H]nicotine was present at 5 nM. (◇) nicotine; (△) imidacloprid; (▽) α-bungarotoxin; (■) QNB. (b) Displacement of [^{3}H]QNB binding to homogenates of *B. microplus* larvae by muscarinic and nicotinic antagonists and agonists. [^{3}H]QNB was present at 2·5 nM. (■) QNB; (●) atropine; (▼) pirenzepine; (◆) 4-DAMP; (▲) methoctramine; (◇) nicotine; (○) d-tubocurarine; (△) imidacloprid; (▽) α-bungarotoxin. $n = 3$. (c) Displacement of [^{3}H]QNB binding to homogenates of *C. tentans* cells by muscarinic and nicotinic antagonists and agonists. [^{3}H]QNB was present at 2·8 nM. (■) QNB; (●) atropine; (▲) oxotremorine M; (▼) carbachol; (◇) nicotine; (△) imidacloprid; (▽) α-bungarotoxin. (d) Displacement of [^{3}H]QNB binding to homogenates of *B. microplus* larvae by muscarinic agonists and receptor desensitization. [^{3}H]QNB was present at 2·3 nM. (■) QNB; (●) oxotremorine M; (◆) carbachol; (○) oxotremorine M in presence of 1 mM γ-*S*-GTP.

plus mAChR with mAChR from various arthropod and vertebrate sources.

The biological efficacy of muscarinic compounds is shown in Table 2. Injection of the compounds into adult ticks, as well as contact of larvae with the test compound over a period of seven days, resulted in total mortality for oxotremorine M at the highest concentration tested, with IC_{50} values of 13·9 μg per tick and 4 mg ml^{-1}, respectively in larvae packet tests. Of the cholinergic antagonists tested, only the nicotinic compound, *d*-tubocurarine, showed an effect in both tick injection and tick larvae packet assays. The muscarinic antagonist 4-DAMP was fully active only in the tick injection assay, with an IC_{50} value of 14·2 μg per tick. At the concentrations tested, only the insecticidal nicotinic agonist imidacloprid revealed a high potency

TABLE 2

Displacement of [^{3}H]QNB Binding to *Boophilus microplus* Larvae and *Chironomus tentans* Cell Homogenates and Biological Efficacy of Cholinergic Compounds

Compounds	*IC_{50} (Bioassays)*			*IC_{50}([^{3}H]QNB-binding assays)*	
	B. microplus *adults*[a] (*μg per tick*)	B. microplus *larvae*[b] (*mg ml^{-1}*)	L. cuprina *larvae*[c] (*μg ml^{-1}*)	*Homogenate of* B microplus *larvae* μM (±*SEM*)	*Homogenate of* C. tentans *cells* μM (±*SEM*)
Imidacloprid	⩾20	6·6	5·0	⩾1000	⩾1000
α-Bungarotoxin	⩾20	>10	⩾100	⩾1000	⩾1000
Tubocurarine	16·8	10·2	⩾100	⩾1000	⩾1000
Atropine	⩾20	>10	⩾100	0·29 (±0·09)	7·59 (±3·12)
RS-QNB	⩾20	⩾10	⩾100	0·05 (±0·02)	0·39 (±0·04)
4-DAMP	14·2	>10	⩾100	49·9 (±9·13)	>1000
Methoctramine	⩾20	⩾10	⩾100	121 (±14·2)	>1000
Pirenzepine	⩾20	>10	⩾100	2·13 (±1·02)	>1000
Oxotremorine M	13·9	4·0	⩾100	71·3 (±19·6)	332 (±72·4)
Carbachol	⩾20	>10	⩾100	253 (±87·1)	>1000
Bethanechol	⩾20	>10	⩾100	nd	>1000

[a] Injection of up to 20 μg of compound into fed adult female *B. microplus* ticks. No inhibition of egg laying in control ticks injected with 1 μl DMSO alone.

[b] Filter packet contact assay with *B. microplus* larvae. Treatment for seven days with up to 10 mg ml^{-1} compound (20 mg in 2 ml olive oil 100 cm^{-2} filter, e.g. 0·2 mg cm^{-2}). Control mortality was <0·5%.

[c] Treatment of *L. cuprina* 1st- to 3rd-instar larvae on horse meat with up to 100 μg compound g^{-1} meat. Control mortality of emerging adults < 1%. In case of efficacies >50%, IC_{50} values of bioassays were determined by probit analyses. IC_{50} values of QNB-binding assays (±SEM) were determined by four-parameter logistic curve fitting with Sigma Plot (Jandel Scientific). ⩾ signifies that IC_{50} is >10 times and > up to 10 times greater than highest concentration tested. nd = not determined.

TABLE 3

[^{3}H]QNB Binding Parameters from *Boophilus microplus* Larvae and Other Arthropod and Vertebrate Organisms[a]

Organism	K_D (*equ.*, nM)	K_{ass} (M^{-1} *min*$^{-1}$)	K_{diss} (*min*$^{-1}$)	K_D (*kin.*, nM)
Boophilus microplus	1·20	2·89 × 10^7	2·87 × 10^{-2}	0·99
Chironomus tentans	1·36	4·48 × 10^7	9·08 × 10^{-2}	2·03
Rhizoglyphus echinopus	0·47	4·68 × 10^7	1·92 × 10^{-2}	0·41
Musca domestica	2·4	2·52 × 10^6	7·20 × 10^{-3}	2·8
Periplaneta americana	8·0	7·8 × 10^6	1·44 × 10^{-2}	1·9
Locusta migratoria	0·8	1·32 × 10^8	5·94 × 10^{-2}	0·5
Drosophila melanogaster	0·7	1·20 × 10^8	1·80 × 10^{-2}	0·2
Apis mellifera	0·47	1·33 × 10^7	6·30 × 10^{-3}	0·47
Guinea pig	0·4	4·02 × 10^8	1·20 × 10^{-2}	0·03
Chick	0·12	1·38 × 10^8	9·00 × 10^{-3}	0·06

[a] Table modified after Huang and Knowles[10] and Lummis and Sattelle.[37] Tissue source and reference: *C. tentans* cells (for comparison K_{ass} was recalculated according to the formula $K_{ass} = (K_{obs} - K_{diss})\ L_T^{-1}$ (K_{obs}: slope of the linearized association curve; L_T: concentration of [^{3}H]QNB) and revealed the K_{ass} from [K_{obs} (0·61 min^{-1}) − K_{diss} (9·08 × 10^{-2} min^{-1})] [[^{3}H]QNB (11·5 nM)]$^{-1}$),[17] *M. domestica* heads,[38] *P. americana* nerve cords,[37] *L. migratoria* head ganglia,[29] *D. melanogaster* heads,[30] *R. echinopus* whole mites,[10] *A. mellifera* heads,[39] guinea-pig ileum[40] and chick brain.[41] K_D (equ.) = experimentally determined equilibrium dissociation constant. K_{ass} = association rate constant. K_{diss} = dissociation rate constant. K_D (kin.) = equilibrium dissociation constant calculated from K_{diss}/K_{ass}.

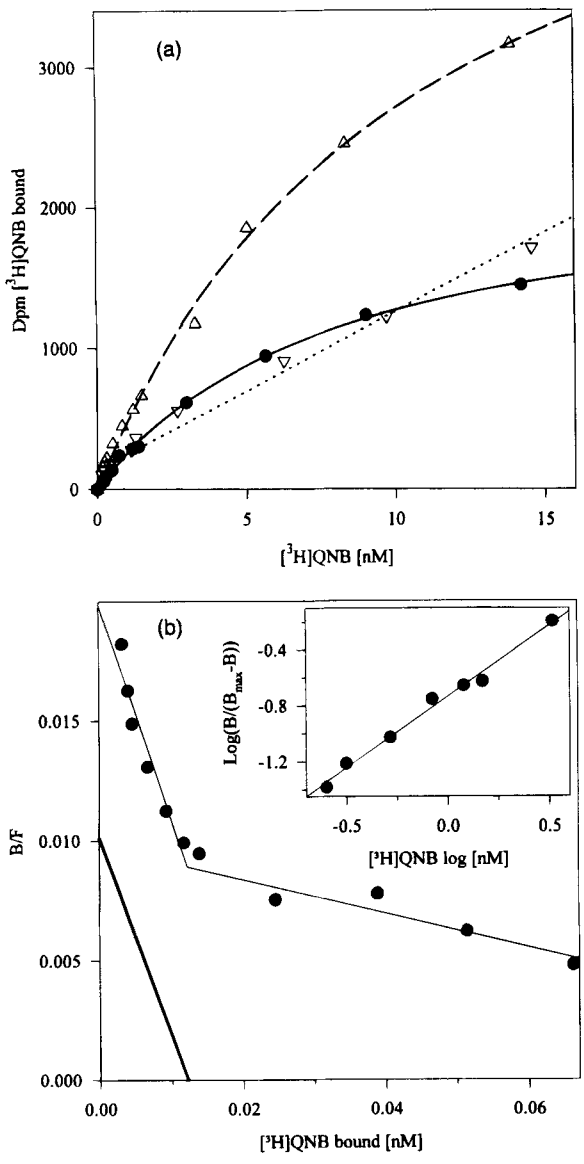

Fig. 2. Scatchard and Hill analysis of [^{3}H]QNB binding to *Boophilus microplus* homogenates. (a) Concentration dependency of specific QNB-binding with 0·1 to 50 nM [^{3}H]QNB ± 1000-fold excess QNB was determined in a binding assay as described in Experimental Methods. Concentrations of radioligand actually present were determined in the incubation vials. Concentrations of specific binding were calculated from the mean of the radioligand concentrations present in the total and non-specific incubation sets. (△) total binding in presence of radioligand at concentration indicated; (▽) non-specific binding in presence of radioligand and 1000-fold excess unlabelled ligand (coefficient of correlation of the least-square fitted straight line is 0·983); (●) specific binding. (b) Transformation of data according to Scatchard.[23] Bold straight line shows data of the high-affinity QNB binding site corrected according to Chamness and McGuire.[24] Coefficient of correlation of least-square fitted straight line is 0·920. Inset shows transformation of data as Hill plot. Slope of the regression straight line and Hill coefficient is 1·021. Coefficient of correlation of least-square fitted straight line is 0·992.

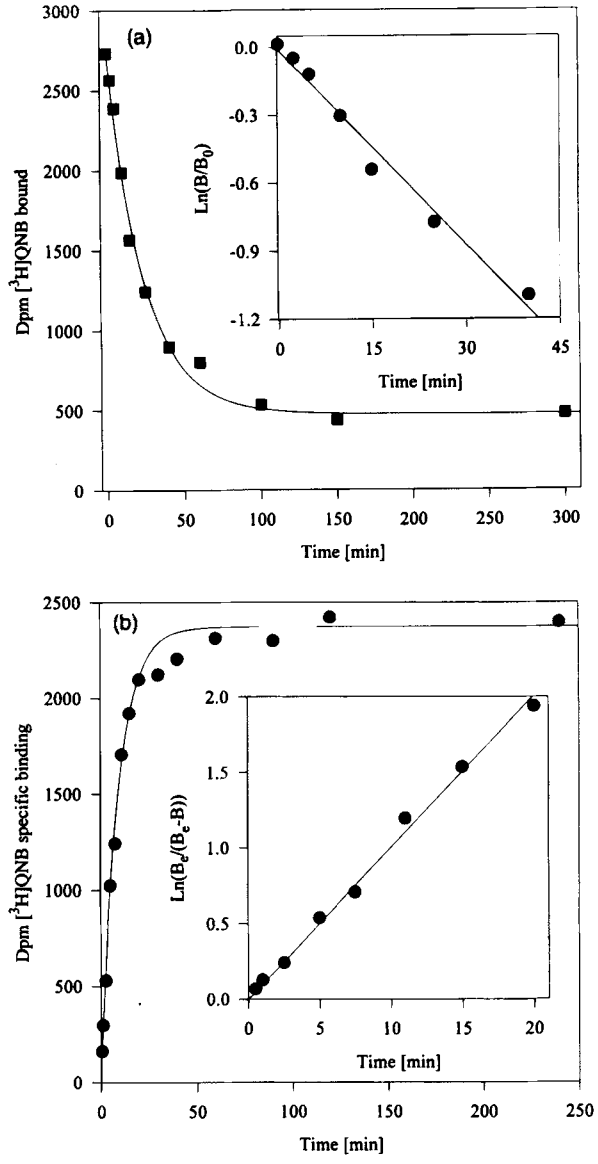

Fig. 3. Determination of rate constants with [^{3}H]QNB binding to *Boophilus microplus* larvae homogenates. (a) Time-course of dissociation of bound [^{3}H]QNB from *B. microplus* membranes. Pre-equilibrated (45 min, 0°C, 2·7 nM [^{3}H]QNB) membranes were supplemented with 100 μM QNB, mixed, filtered and measured as described in Experimental Methods. Inset: A linear transformation of the initial decrease data assuming first-order dissociation kinetics. B is the bound [^{3}H]QNB retained at the indicated time, B_0 the total [^{3}H]QNB bound at time 0. Correlation of the least-square fitted straight line is 0·982. The slope is calculated to be $-0{\cdot}0287(\pm 0{\cdot}0014)\ \text{min}^{-1}$ which corresponds to K_{diss}. (b) Association of the [^{3}H]QNB *B. microplus* membrane complex. Membranes were incubated (0°C) with or without 100 μM QNB, mixed, filtered and measured as described in Experimental Methods. Only specific binding is presented. Inset: A linear transformation of the time-course of specific [^{3}H]QNB binding. B_e is the binding at equilibrium. B the binding at the times indicated. The slope of the least-square fitted straight line (coefficient of correlation: 0·995) revealed a K_{obs} of $0{\cdot}1004(\pm 0{\cdot}006)\ \text{min}^{-1}$. K_{ass} is calculated according to the formula $K_{ass} = (K_{obs} - K_{diss})/L_T$ (L_T: concentration of [^{3}H]QNB = 2·475 nM).

against larvae of *Lucilia cuprina* Wied. with an IC_{50} value of 5 $\mu g\ ml^{-1}$. Even at 20-fold higher concentrations, no mortality was observed with any of the other cholinergic compounds (Table 2). No significant effect in mAChR contents, acetylcholine esterase activity or morphological appearance was found upon treatment of *C. tentans* cells with muscarinic agonists (carbachol, oxotremorine M, bethanechol), atropine or imidacloprid (data not shown). Binding assays with homogenates from *C. tentans* cells revealed that oxotremorine M (IC_{50} 332 μM) and atropine (IC_{50} 7·6 μM) at least compete for [^{3}H]QNB binding (Fig. 1(c)).

4 DISCUSSION

B. microplus is sensitive to treatments with muscarinic agonists.[9] A binding protein for cholinergic compounds has to be characterized in the cattle tick as a prerequisite for the testing of large numbers of compounds in receptor binding studies and the larval stage in tick development was chosen for this study. Prior to feeding, larvae do not contain contaminating blood from the host which could have compromised our experiments. On the other hand, treatment of larvae is more important from a veterinary point of view, since this will prevent the establishment of an infestation with fully sucking adults of the one-host tick *B. microplus*. From the binding experiments with various radioligands and non-labelled cholinergic compounds it is concluded that the predominant binding site present in the larvae of the cattle tick *B. microplus* is of muscarinic pharmacology. The muscarinic ligand [^{3}H]QNB is displaced only by ligands of muscarinic specificity. The nicotinic agonists imidacloprid and nicotine and the nicotinic antagonist α-bungarotoxin are not able to compete for [^{3}H]QNB binding in cattle tick larvae homogenates. At the same time, no significant specific binding is found for [^{3}H]imidacloprid in concentrations used for insect nAChR binding assays.[25] A high-affinity nicotinic binding site was detectable in tick larvae homogenates with [^{3}H]nicotine. Imidacloprid and the muscarinic antagonist QNB have only low capacities to displace [^{3}H]nicotine binding. This might suggest a low-affinity binding site for imidacloprid in homogenates of *B. microplus* larvae which corresponds to the weak biological efficacy of imidacloprid against ticks. In contrast to these findings, imidacloprid displays a strong biological efficacy in insects, and a corresponding high affinity to its acetylcholine receptor binding site.[5,25,26]

In contrast to the non-neuronal insect cell system, it is reasonable that homogenates of whole cattle tick larvae should contain both neuronal and non-neuronal AChR receptors. For the insect central nervous system, at least three populations of binding sites are known which display solely nicotinic, solely muscarinic and mixed nicotinic-muscarinic pharmacology.[27,28] From the data presented here, it can be concluded that muscarinic acetylcholine receptors are present in homogenates of *B. microplus* larvae. Additionally, there is evidence for a nicotinic binding site with 100- to 1000-fold higher affinity for the nicotinic than for to the muscarinic compounds tested.

As can be seen from Table 3, association and dissociation rate constants of the [^{3}H]QNB receptor complex, as well as the equilibrium dissociation constant, are of the same order of magnitude as those for *Locusta migratoria* L. and *Drosophila melanogaster* Meig. neuronal tissues and homogenates from the bulb mite *Rhizoglyphus echinopus* Fum. + Rob.[10,29,30] For *L. migratoria*, *D. melanogaster* and *R. echinopus* the Hill coefficients were 0·97, 1·02 and 1·0, respectively, which indicates non-cooperative binding.[10,29,30] A Hill coefficient of 1·02 was also determined for the mAChR from *B. microplus* larvae. The B_{max} value of 22·5 fmol mg^{-1} protein is two- to four-fold lower than those for mAChR from *L. migratoria* and *D. melanogaster* and in the same range as that for mAChR from the nematode *Caenorhabditis elegans* Maupas (15·6 fmol mg^{-1} protein).[29–31] For the bulb mite, a B_{max} value per mg protein has not been reported, but a B_{max} value of 202 fmol g^{-1} wet weight was determined.[10] Using an estimate for the tick larvae wet weight, the B_{max} value for mAChR of *B. microplus* larvae corresponds to 900 fmol g^{-1} wet weight, which is about 4·5-fold higher than the value reported for the bulb mite. The following explanations can be given for the discrepancy between the two members of the acari. (1) The *B. microplus* homogenates have been prepared from one stage in the life cycle, whereas *R. echinopus* homogenates were prepared from a colony which represented adults, larvae and nymphs of different nutritional status.[10] Assuming that larvae have a relatively high titre of mAChR, a homogenate from a mixed population might have led to a lower proportion of larval stages. (2) The bulb mites were taken from a colony maintained on artificial medium,[10] whereas the tick larvae did not feed on cattle. The medium taken up by the mite population might lead to an overestimation of weight, which will also give an explanation for the relatively low mAChR titre in the mites. (3) Different types of assays for separation of bound and free ligands were used for the determination of mAChR in the two acari.

Since nematode, bulb mite and tick larvae have to be prepared as whole animal homogenates, the B_{max} values correlated to protein contents are lower because of the relatively low proportion of neuronal tissue compared to head or ganglia homogenates from larger insects. Generally, the mAChR titre in insect tissues is much lower than that in vertebrates.[30]

Binding studies involving a non-hydrolysable GTP analogue revealed a 4·8-fold reduction of mAChR affinity to the muscarinic agonist oxotremorine M. This indicates that the mAChR binding site in *B. microplus*

larvae is a G-protein-coupled receptor. Similar effects have been described for *C. tentans* cells and for *L. migratoria*.[18,32]

Displacement curves revealed 50% inhibition values (summarized in Table 2). For comparison the IC_{50} values can be converted into K_i values.[21] The sequence of K_i values for the subtype-specific antagonists pirenzepine, 4-DAMP and methoctramine is 0·69 μM, 16·2 μM and 39·4 μM, respectively. A similar sequence was reported for bulb mite mAChR, revealing K_i values of 0·033 μM for pirenzepine and 34 μM for methoctramine; 4-DAMP, however, was not tested. The mAChR found in lobster also binds pirenzepine with higher affinity than 4-DAMP followed by methoctramine, with K_i values of 0·43 μM, 0·8 μM and 4·3 μM.[33] Insect mAChR mainly show higher affinity for 4-DAMP, followed by pirenzepine and M2-specific antagonists like methoctramine or AF-DX 116.[34] The discriminating compounds have been used to describe the pharmacology of vertebrate mAChR subtypes and indicate that similar subtype specificities also occur in invertebrates. Whether there is a specific pharmacology for invertebrate mAChR depending on the tissue or developmental stage has yet to be elucidated.

The AChR of *C. tentans* previously described as of solely muscarinic specificity with no binding of α-bungarotoxin[17] has been additionally tested for imidacloprid binding sites. Since imidacloprid generally shows a more pronounced binding to insect cholinergic receptors than to vertebrate nicotinic binding sites if compared to α-bungarotoxin, it could be stressed that imidacloprid would be a better ligand for the demonstration of nicotinic binding sites.[25,26,35] Therefore, we confirmed binding data for QNB and other cholinergic compounds in homogenates from the epithelial *C. tentans* cell line. Additionally, binding experiments with tritiated imidacloprid revealed no high-affinity binding sites for this compound in the epithelial cell line which was already designated to be of non-neuronal origin.[16] Conversely, imidacloprid did not displace binding of [^{3}H]QNB to cell homogenates. There were no detectable alterations in key enzyme activity or receptor density or in the morphological appearance (e.g. form, size, density) of the *C. tentans* cells upon treatment with any of the cholinergic ligands. Whole-cell binding assays with tritiated QNB have been performed successfully.[17] Since only muscarinic binding sites seem to be present in the insect cell line, a nicotinic response is not expected. Yet not all possible effects of muscarinic agonists on the insect cell line have been investigated. Recent work on apoptosis in the vertebrate neuronal cell line PC12 expressing a M1 mAChR revealed that muscarinic agonists like oxotremorine M or carbachol prevent cell death upon depletion of nerve growth hormone.[36] *C. tentans* cells respond to incubations with physiological concentrations of 20-hydroxyecdysone with changes in enzyme activities and cell morphology. Since oxotremorine M binds to the *C. tentans* mAChR, possible effects of this muscarinic agonist on treatment of the insect cells with the moulting hormone 20-hydroxyecdysone are currently under investigation.

For the first time an AChR of clearly muscarinic nature has been characterized in homogenates of cattle tick larvae. Biological efficacy of muscarinic agonists was demonstrated but none fulfilled our criteria for highly active compounds. Since the biological results roughly parallel the receptor binding data, the receptor binding assay can be used for screening large numbers of related and unrelated compounds prior to biological testing. A binding assay with mAChR from the *C. tentans* insect cell line will focus mainly on mAChR involved in differentiation and development, and should give information on insecticidal compounds which are likely to have new insect growth regulator properties.

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